STUDY ON THE HUMAN COAGULATION FACTOR IX PROMOTER

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ABSTRACT

Fresh blood specimens were obtained from a normal subject and a haemophilia B patient for DNA extraction. The blood samples were centrifuged to collect the lymphocytes and leucocytes which were lysed with sodium dodecyl sulphate. After enzyme digestions by Proteinase K and RNase, DNA was extracted by subsequent treatment with salt saturated phenol and phenol/chloroform (1:1). Genomic DNA was then precipitated by isopropanol and spooled out with a sealed pasteur pipette. The purity and quantity of DNA was determined by uv spectrophotometry and agarose gel electrophoresis.

A polymerase chain reaction (PCR) was established for amplification of a DNA fragment of the promoter region of the Factor IX (FIX) gene using the extracted genomic DNA as templates. Two pairs of 3′ and 5′ oligonucleotides flanking the FIX gene promoter were used. I optimized the PCR conditions including primer concentrations, template concentrations, $MgCl_2$ concentration, and temperature programme. The DNA products amplified by PCR were then purified by three methods: glassmilk adsorption using a commercially supplied GENECLEAN Kit, glasswool exclusion method and extraction from polyacrylamide gel after electrophoresis. By the first two methods, I obtained very poor yield and the DNA materials were fragmented after many

trials. Only the third method produced DNA samples that could be used for subsequent experiments.

Direct sequencing of the PCR DNA was attempted using a commercially supplied ^{35}Sequencing kit. Only very weak signals were obtained after several trials. Another protocol utilizing the dideoxynucleotide termination method was attempted using reagents made up by myself. Slightly better signals were obtained but still no convincing readable sequence could be produced. Attempts to clone the PCR DNA into M13 phage vector for transformation in competent cells were unsuccessful.

Because of the time constraint and of other limitations in facilities of my laboratory which was newly established for molecular cloning, experiments described above were not proceeded further. The cloning experiments should be produce adequate amount of the FIX DNA by PCR for elucidation of the sequence.

1. INTRODUCTION

Human Blood Clotting Cascade

The human blood clotting reactions involve three major steps: Firstly, an initial step in which an activator is generated that activates prothrombin. A second step in which this prothrombin activator converts prothrombin to thrombin, then followed by the third step in which thrombin converts fibrinogen to fibrin [1].

Two clotting systems for converting prothrombin to thrombin, the intrinsic and extrinsic systems, have been described [2] (Fig. 1). The intrinsic clotting reactions involve the factors XII, XI, IX, VIII, X, and V. Factors XII and XI involved in the surface activation. Factors V, VIII, and I are altered during clotting by thrombin.

The extrinsic clotting reactions involve the factors VII, X, and V which give rise to an extrinsic prothrombin activator. Factor VII, is unique for the extrinsic clotting reactions. Tissue thromboplastin by-passes the need for factors XII and XI and forms a calcium-dependent complex with factor VII, which activates factor X. In this extrinsic activation of factor X, the protein portion of the tissue thromboplastin molecule substitutes for factor VIII; and factor VII substitutes for factor IX. Thus, the combination of tissue

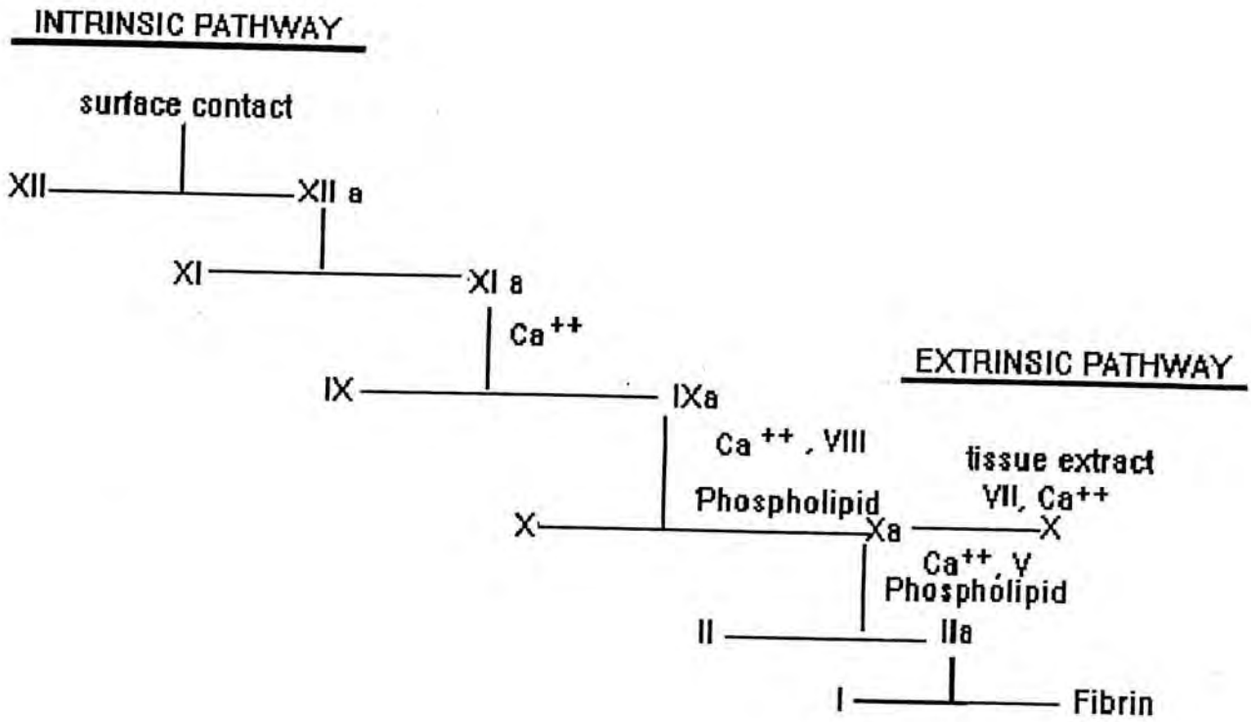

Fig. 1 The human clotting cascade showing the intrinsic and extrinsic clotting systems.

thromboplastin and factor VII bypasses the requirement for the two antihaemophilic factors: factors VIII and IX.

Blood Clotting Disorders

Hereditary bleeding disorders caused by reduced clotting activity of each of the plasma clotting factors have been identified [3]. These disorders may stem from two types of genetic defects: a mutation that prevents synthesis of a clotting protein (for example, failure to synthesize factor VIII in von Willebrand's disease), or a mutation that causes synthesis of an abnormal molecule with impaired clotting activity (for example, the abnormal factor VIII molecules lacking factor VIII clotting activity in haemophilia A). The patient with factor XII deficiency may have no history of abnormal bruising or bleeding, even after surgery, but has an extremely prolonged clotting time in a glass tube, more prolonged than blood specimens from many patients with severe haemophilia. The haemophilias, factor XI deficiency, and factor XII deficiency affect intrinsic clotting, whereas factor VII deficiency impairs only extrinsic clotting. Prothrombin deficiency, factor X deficiency, and factor V deficiency affect both intrinsic and extrinsic clotting.

Haemophilia A (factor VIII deficiency) and haemophilia B (factor IX deficiency) have similar clinical features and clotting abnormalities in screening tests [4]. The clinical

hallmarks of both haemophilias A and B are (1) lack of excessive haemorrhage from minor cuts or abrasions, because platelet function is normal, (2) joint and muscle haemorrhages leading to difficult and disabling long-term sequelae, (3) easy bruising, and (4) prolonged and potentially fatal post-operative haemorrhage. In general, a prolonged whole blood clotting time, prolonged activated partial thromboplastin time (APTT), and an elevated level of residual serum prothrombin may suggest that the patient is a haemophiliac. To confirm the case, further investigation of clotting factors including the measurement of factors VIII and IX activity as well as their antigen levels is available in most hospital. A more detailed classification of haemophilia variant which requires techniques of molecular genetics is seldom done.

Haemophilia A is an X-linked recessive bleeding disorder attributable to decreased blood levels of functioning procoagulant factor VIII. This disorder accounts for approximately 80% of true haemophilas [5].

Haemophilia B is also an X-linked recessive bleeding disorder which can occur in a severe, moderate, or mild form, depending on the level of factor IX (FIX) activity in plasma [6,7]. The severe form has a FIX level of less than 1% of normal; almost half of the haemophilia B patients belong to this group [8]. The clotting time in these

patients is usually longer than 1 hour. In moderate haemophilia B case, the FIX level is 1% to 4%, and the clotting time is about 20 to 30 minutes. The FIX activity in mild haemophilia is 5% to 25% of normal; these patients have slightly prolonged clotting times. Lower levels of FIX have been found in carriers of haemophilia B. Patients with mild deficiency of FIX (generally have greater than 5% factor activity) have little spontaneous bleeding. The incidence of haemophilia B is 1 in 30,000 Caucasian males [9].

Biochemistry of Factor IX

Human coagulation FIX is also called anti-haemophilic factor , Christmas factor , or plasma thromoplastic component (PTC) . It is a vitamin K-dependent single-chain glycoprotein that participates in the intermediate stage of blood coagulation [5]. It is synthesized in the liver and circulates in the blood as a zymogen which is cleaved by serine protease. Factor IX contains twelve -carboxyglutamic acid residues (Gla). The Gla residues play an important role in calcium binding in the clotting process. They can also be found in other vitamin K-dependent clotting factors such as factors II, VII and X, and proteins C and S. The molecular weight of FIX is 55,000 daltons and the biological half-life is about 18 to 24 hours.

FIX can be activated either by factor XIa or factor VIIa plus tissue factors to form activated factor IX (FIXa). In the presence of Ca^{++} , factor VIII and platelet phospholipid molecules, FIXa can form a "factor X converting complex". This complex converts factor X into factor Xa and ultimately results in the formation of the fibrin clot [10].

Structure of the Factor IX Gene

The human FIX gene is located at locus Xq 27.3 on the distal long arm of the X chromosome and is primarily expressed in the liver [11]. It is 34 kbases long with eight exons and seven introns. Both the FIX gene and the cDNA copy of factor IX mRNA have been cloned and sequenced [12,13,14]. The 8 exons have been shown to encode separate domains in the FIX protein (Fig. 2) which are: (a) a hydrophobic signal peptide for controlling protein secretion from the hepatocyte into the blood stream; (b) a propeptide and Gla domain for post-translational Γ-carboxylation of 12 glutamic acid residues in the Gla region; (c) a small hydrophobic domain for promoting heterodimerisation of Gla domains of unrelated clotting factors [15]; (d) a type B, epidermal growth factor-like domain, which is homologous to epidermal growth factor (EGF). This domain binds an additional Ca^{2+} with high affinity and may also bind to factor VIII. (e) A type A, epidermal growth factor-like domain which is similar to exon d but lacks the homologous carboxylate residues; (f) the activation domain includes the 35-residue peptide that is

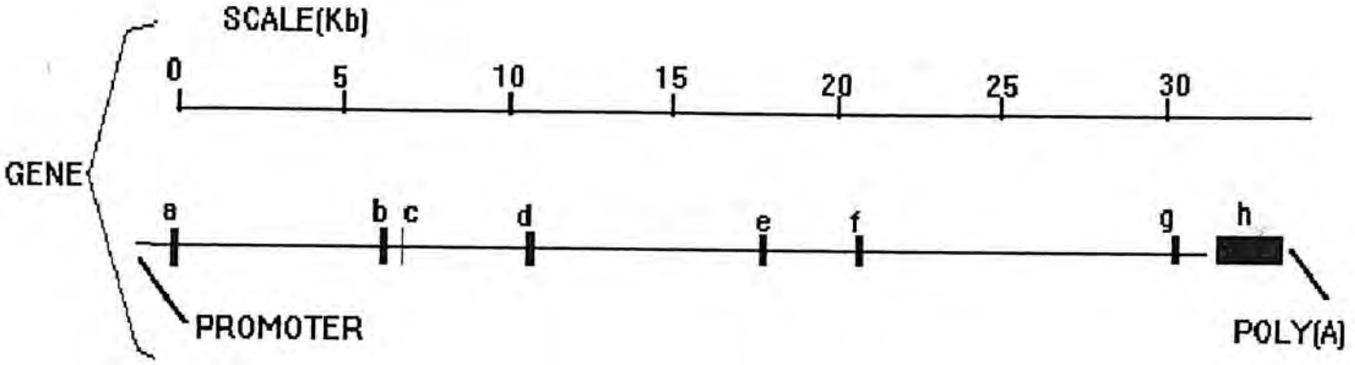

Fig. 2 The human factor IX gene showing the eight exons, the introns, position of the promoter and the poly (A) site.

removed during zymogen activation, (g) and (h) the serine protease domain, responsible for the proteolysis. The active site of FIX is contained within the serine protease domain, which is supported by 6 other signalling regions. The active site His -221 is in exon g, but the other two active site residues, Asp -269 and Ser -365 residues of the charge relay system of the active site, and Asp -359 of the substrate binding pocket, are all in exon h. This type of arrangement is different from those other serine proteases. The eight domains mentioned above play different roles which are essential either for biosynthesis, modification, activation by XIa or for ensuring maximal catalytic activity [12].

Molecular Defects of the Factor IX Gene

There is a wide variation in terms of molecular defect in haemophilia B patients [16]. For patients catergorised as antigen positive, point mutations in the FIX gene coding region resulting from mild, moderate, and severe bleeding disorder. In the antigen negative group, either deletions or point mutations for the correct biosynthesis of messenger RNA or the protein is affected severely. Point mutation is characterized by a single base pair change, it may be a base substitution, a base insertion, or a base deletion. A database with 221 entries lists all known point mutations and short deletions and additions in the FIX gene has been established [17]. Of the 216 known mutations within the

gene, 115 are unique molecular events, the remainder being repeats. Most of the repeats occur at CG dinucleotides and involve a CG→TC or CA change which can be considered as 'hotspots'. Mutations have been detected in all domains and control regions within the gene, except the signal peptide and poly(A) site. On the basis of prolonged ox brain prothrombin time measurement, a haemophilia Bm variant is identified [18].

A rare type of haemophilia B, the Leyden form, has also been described [19,20]. Patients with this type of bleeding disorder show severe symptoms in childhood. However, FIX level is rising with increasing age and progressing to a mild or an asymptomatic condition after puberty. The FIX level may rise from less than 1% in early childhood before puberty to more than 50% of normal after puberty. Another less severe form of haemophilia B Leyden, namely, haemophilia B High Wycombe, with different G→A mutation at -6 has also been described [21]

The use of polymorphic DNA markers usually can determine the parental source of each X chromosome [22,23]. Restriction fragment length polymorphism (RFLP) with intragenic oligonucleotide markers has been used for diagnosis of carrier state and claimed to be with 99.9% reliability [24]. With intragenic RFLPs, the results obtained are essentially

qualitative and are easily interpretable for both carrier detection and prenatal diagnosis. However, important differences have now been shown between the Chinese and Caucasian populations. None of the four RFLPs (Taq_I, Dde_I, Xmn_I, and Msp_I) can be used for family studies and prenatal diagnosis in the Chinese population of Jiangsu province [25].

The advent of polymerase chain reaction (PCR) technique makes it possible to amplify the genomic DNA for higher yield of product to be able to perform direct sequencing [26]. Substitution of Cys 336 and Asn 120 as well as deletion of Arg 37 lead to the decreased level of FIX antigen, whereas in the substitution of Arg -4, Arg 333, Asp 64 and Pro showed no significant reduction of protein serum levels. These abnormalities are identified by direct sequencing of amplified genomic DNA which overcome the limitation of heterozygote and marker required in RFLP [16] . Other methods such as dimethyl sulphoxide and amplification and mismatch detection have been introduced to improve direct sequencing and thus enhance the intensity of signal [27,28].

The Factor IX Promoter

Mutations in promoters affect the level of expression of the gene(s) they control, without altering the gene products themselves. Almost all of the point mutations that affect

promoter function fall within the two consensus sequences which located at -35 and -10 with consensus sequences TTGACA and TATAAT respectively [29]. It has been demonstrated that a consensus TATA box, and sometimes a CCAAT box [30] and further upstream sequences are significant for eukaryotic promoters. However, neither TATA box nor CCAAT are observed in the FIX promoter [13]. All cases of haemophilia B Leyden reported in the database of point mutations [17] show abnormality in the promoter region, therefore it is a region of interest in FIX gene study. More new mutations are expected to be found in the FIX promoter, particularly in non-Leyden haemophilia B patients.

Although there is no report on incidence rate of haemophilia B in Chinese, reports of studies on FIX deficiency in Jiangsu Province suggested a possible high frequency in that area, which is situated in the middle part of China. However, haemophilia B is not regarded as prevalent in Hong Kong, where the population is mostly Southern Chinese. Moreover, studies on the FIX promoter in Chinese has not been reported. I intend to investigate the promoter sequence in a Chinese haemophilia B patient born in Hong Kong.

2. OBJECTIVE

To establish protocols of DNA extraction, polymerase chain reaction and sequencing for comparison study of the FIX promoter sequence of a haemophilia B patient and a normal subject.

3. MATERIALS AND METHODS

3.1 Materials

3.1.1 Enzymes

1. Protease (Proteinase K) Type XXVIIII from Tritirachium album (Sigma)
2. Ribonuclease A (Ribonuclease I; EC 3.1.27.5) Type XII-A: from Bovine Pancreas (Sigma)
3. Taq DNA polymerase (Pharmacia)
4. T4 DNA ligase (Amersham)
5. Alkaline phosphatase (calf intestinal mucosa) (Pharmacia)
6. Restriction Endonuclease Sma I (Amersham)
7. Klenow fragment of E.coli DNA polymerase I (Pharmacia, provided in the ^{35}Sequencing Kit)

3.1.2 DNA Markers

1. Hae III Digest ϕX-174-RF DNA (Pharmacia)
2. DNA Hind III Digest (Pharmacia)
3. PATX $Hinf_1$ (self-prepared)
4. Hae III Digest pBR322 DNA (Sigma)

3.1.3 General Reagents

Acrylamide (Sigma)

Agarose (Sigma)

Amberlite, Mixed Bed Resin TMD-8 (Sigma)

Adenosine triphosphate (ATP)

Bovine serum albumin (Sigma)

Bromphenol blue (Merck)

Chloroform (BDH)

Dideoxynucleotide triphosphate (ddNTP) set 5mM solutions (Pharmacia)

Deoxynucleotide triphosphate (dNTP) set 100mM solutions (Pharmacia)

Dimethyl sulphoxide (DMSO)

Dithiothreitol (Merck)

Ethidium bromide (Sigma)

Ficoll (type 400) (Pharmacia)

Formamide (Sigma)

8-Hydroxyquinoline (Sigma)

Isopropanol (Merck)

Isopropyl ß-D-Thiogalactopyranoside (IPTG) (Sigma)

2-Mercaptoethanol (Merck)

N,N-Methylene diacrylamide (Merck)

N,N,N',N'-tetramethyl ethylenediamine (TEMED) (Sigma)

Paraffin liquid (Merck)

Phenol (Merck)

Trizma base, Purity 99.9% (Sigma)

Trizma hydrochloride (Sigma)

Xylene cyanol FF (Sigma)

X-Gal (5-bromo-4-chloro-3-indolyl-ß-D-galactoside) (Sigma)

Other reagents are either of Biochemical or Anala R grade from Sigma, BDH or Merck.

3.2 General Methods

3.2.1 Preparation of Phenol and Phenol/Chloroform (1:1)

Phenol (400 g) was dissolved in 400 ml 0.1 M Tris buffer, pH 8.0, by constant stirring. The upper aqueous layer of Tris was then aspirated, and hydroxyquinoline was added to the organic phase to give a final concentration of 0.1% (w/v). Equal volume of 0.1 M Tris buffer, pH 8.0 was added, the mixture was stirred and the upper aqueous layer was then aspirated. This step was repeated five times. Finally, a 0.2% ß-mercaptoethanol in 1:10 (v/v) Tris, pH 8.0, was added and the liquid phenol was stored in an amber bottle at 4°C for up to one month.

For the preparation of 1:1 phenol/chloroform, 400 g phenol was dissolved in 400 ml 0.1 M Tris pH, 8.0 and hydroxyquinoline was added. Equal volume of chloroform as the phenol layer was added. The mixture was stirred and the upper aqueous layer aspirated. This procedure was repeated five times. Finally, ß-mercaptoethanol was also added and the solvent mixture was stored in an amber bottle at 4°C.

3.2.2 Buffer preparation

10 X TBE Buffer, pH 8.4: 0.9 M Tris

0.89 M Boric acid

25 mM EDTA

50 X TAE Buffer, pH 8.0:	2 M Tris base
	5.7% Acetic acid
	50 mM EDTA
TE Buffer, $TE_{0.1}$:	10 mM Tris, pH 8.0
	0.1 mM EDTA
$TE_{1.0}$:	10 mM Tris, pH 7.4
	1m M EDTA
RNase Buffer pH 7.4:	10 mM Tris
	15 mM NaCl
Gel-loading Buffer:	0.25% Bromphenol blue
	0.25% Xylene cyanol FF
	15% Ficoll
ACD Solution:	23 mM Citric acid
	45 mM Sodium citrate
	82 mM Glucose
Lysing Buffer 1:	100 mM NaCl
	1 mM EDTA
	10 mM Tris, pH 8.0
	0.5% SDS

Lysing Buffer 2:

100 mM NaCl

5 mM EDTA

100 mM Tris, pH 8.0

0.5% SDS

T4 Ligase Buffer:

300 mM Tris, pH 7.8

100 mM $MgCl_2$

100 mM DTT

10 mM ATP

Amplification Buffer:

Stock amplification buffer

0.18 M $(NH_4)_2SO_4$

88 mM $MgCl_2$

0.74 M Tris, pH 8.8

Working buffer (10 X buffer 8)

89 µl amplification buffer

10 µl 10 mg/ml BSA

1 µl ß-mercaptoethanol

This working buffer was prepared freshly for each PCR.

5 X Sequenase Buffer:

200 mM Tris, pH 7.5

100 mM $MgCl_2$

250 mM NaCl

3.2.3 Agarose Gel Electrophoresis

Horizontal agarose gel electrophoresis was carried out on a 'homemade' perspex apparatus. One percent gel was prepared by dissolving 3 g agarose in 300 ml 1 X TBE or 1 X TAE buffer, followed by melting in a microwave oven. After adding 5 µl of 10 mg/ml ethidium bromide, appropriate amount of the gel was poured into an electrophoresis tray for solidification. Wells on the gel were formed with a comb. After solidification and loading of DNA markers and samples, electrophoresis was carried out in an electrophoresis tank. Voltage of 1-5 V/cm was applied until bromphenol blue and xylene cyanol FF were migrated to appropriate distance on the gel. Photograph was taken under uv light on Polaroid film.

3.2.4 Polyacrylamide Gel Electrophoresis (PAGE)

Native polyacrylamide gel was carried out on a Protean II gel apparatus (BioRad, Richmond, CA, USA). Denaturing polyacrylamide gel was carried out on a 'homemade' vertical gel apparatus.

Preparation of Stock Acrylamide Solution

10/9 20% Native acrylamide stock

One hundred and ninety g acrylamide and 10 g N,N methylene bisacrylamide were added into 900 ml distilled water. Deionization was carried out by adding one tablespoonful of Amberlite monobed resin, followed by mixing and filtering.

The 5% Native PAGE gel was prepared:

5 ml 10/9 20% native acrylamide

2 ml 10 x TBE

13.8 ml water

200 μl 10% ammonium persulphate

20 μl TEMED

Preparation of Denaturing Polyacrylamide Gel

A 30% denaturing PAGE gel for sequencing was prepared by dissolving 28.5 g acrylamide and 1.5 g bis-acrylamide in 100 ml deionized H_2O.

To prepare 7 M urea in 5% polyacrylamide gel, 63 g of urea was combined with 15 ml of 10 X TBE and 25 ml of 30% acrylamide stock solution. The total volume was adjusted to 150 ml with distilled water. It was then filtered through an 0.45 micron mesh filter and degassed.

3.3 DNA Extraction

3.3.1 Haemophilia B Patient

The haemophilia B patient WPC is a seventeen years old male patient. When he was at 3 years of age he suffered minor head trauma with persistent oozing from the mouth. Activated partial thrombin time was prolonged to 134 seconds (control 45 seconds) which was corrected by serum, indicating a possible FIX deficiency. However no FIX assay was available

at that time. Since then, he had 4-5 haemorrhagic episodes per year mainly of haemarthrosis, muscle bleeding and gum bleeding occasionally treated by fresh frozen plasma or prothrombin concentrates. One year ago, his APTT was measured again and it was 88 seconds. FIX level was also checked. Unfortunately, the result was somehow unretrievable. Nevertheless, the word "low FIX activity" was recorded in the patient record.

3.3.2 Blood Collection

Five millilitres blood was collected from a male normal subject (CPP) and the haemophilia B patient (WPC) by venous puncture into a sample tube containing 1.7 ml ACD solution.

3.3.3 DNA Extraction

The blood specimens (5 ml whole blood in 1.7 ml ACD) were centrifuged at 3000 X g for 10 minutes. Plasma was discarded by aspiration and about 1.5 ml buffy coat was collected. Lysing buffer 1 (2.5 ml) was added and the mixture was vortexed for 2 minutes. About 1 mg proteinase K was added. The sample mixture was incubated at 55°C for 1 hour then at 37°C overnight under constant shaking. In the next day 0.1 mg RNase was added and the sample mixture incubated at 37°C for another 10 minutes. An equal volume of phenol was added and the sample vigorously vortexed for 10 minutes followed by centrifugation at 8000 X g for 15 minutes. This step was repeated once followed by two extractions by

phenol/chloroform (1:1). DNA was spooled out with sealed pasteur pipette after addition of 2 volumes of isopropanol. The DNA obtained was dissolved in 300 μl $TE_{1.0}$.

If the DNA obtained showed high absorption at 280 nm it would be taken for further digestion by proteinase K and RNase and re-extraction by phenol and phenol/chloroform.

Three DNA samples were prepared from blood samples taken from the normal subject CPP and were labelled as CPP-1, CPP-2 and CPP-3. One DNA sample was prepared from the patient, labelled as WPC-1.

3.3.4 **DNA Quantitation**

DNA was quantitated by uv-spectrophotometry on a Response Spectrophotometer (Gilford, Oberlin, Ohio, USA). Absorption at wavelength 260 nm and at 280 nm was taken. The reading at 260nm allowed calculation of the concentration of nucleic acid in the sample. An optical density (OD) of 1 corresponds to approximately 50 μg/ml of double-stranded DNA, 40 μg/ml single-stranded DNA, and 20 μg/ml single-stranded oligonucleotides. The ratio between the absorbance at 260nm and 280nm (OD_{260}/OD_{280}) provides an estimate of the purity of the nucleic acid.

3.3.5 Polymerase Chain Reaction (PCR)

Polymerase chain reaction was carried out on a PCR TempCycler (Coy, Ann Arbor, Michigan, USA). Four oligonucleotides were kindly provided by Professor George Brownlee, Sir William Dunn School of Pathology, University of Oxford. They were used as primers for both PCR and sequencing of the FIX promoter. Different combinations of primers were tested for annealing. (Fig. 3)

101 -- 5′ upstream sequence

5′-GTGCT GCCAC AGTAA ATGTA-3′

102 -- 3′ downstream sequence

5′-TGATG AGGCC TGGTG ATTCT-3′

103 -- 5′ upstream sequence

5′-GCCTA TCTCC ATTCT GA-3′

104 -- 3′ downstream sequence

5′-GCACT GAGTA GATAT CCTAA AAG-3′

Human genomic DNA from a normal subject -- samples CPP-1, 2 and 3, and a haemophilia B patient -- sample WPC-1, were used as templates.

Optimization of PCR

A series of experiments (PCR-1 to PCR-7) have been carried out to obtain the optimal amplification conditions. The PCR products obtained after each experiment was analysed by 5% (w/v) polyacrylamide gel electrophoresis.

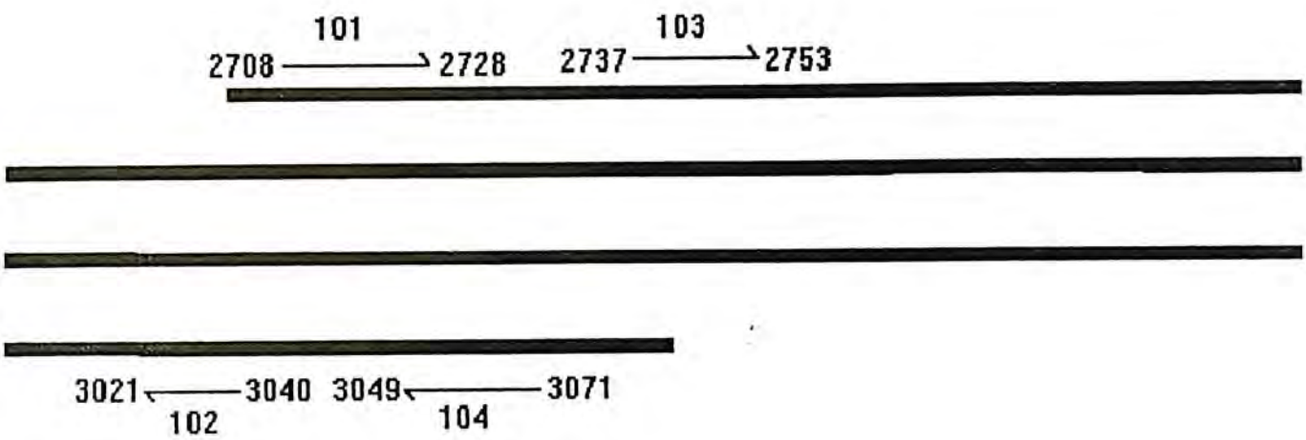

Fig. 3 Position of the 2 pairs of primers, #101 and #102, and #103 and #104 on the FIX promoter sequence. The numbering system is that used in [14]. PCR products obtained from primers #101 and #102 has a length of 332 bp and that from primer #103 and #104 also a length of 334 bp.

PCR-1: Initial set up.

To a 0.5 ml PCR reaction tube was added 5 μl 10 X buffer 8, 5 μl 15 mM dNTP, 2 μl primer 101 (200 ng) and 2 μl primer 102 (200 ng). DNA templates and water were then added.

Sample	DNA Template	Water
PCR-1-1	Nil	36 μl
PCR-1-2	CPP-1: 2 μl (320 ng)	34 μl
PCR-1-3	CPP-1: 5 μl (800 ng)	31 μl

The final volume of each reaction mixture was 50 μl and 50 μl paraffin oil was layered on top to prevent evaporation. After incubation at 95°C for 7 minutes, 1.25 units Taq DNA polymerase 1 μl was added to each sample. The reaction mixture was vortexed gently and centrifuged briefly before being placed into the TempCycler. Sample holding blocks of the TempCycler were filled with glycerol to provide uniform heating. The temperature programme was set as:

Denaturing temperature:	91°C,	1 min
Annealing temperature:	60°C,	1 min
Extension temperature:	65°C,	3 min 10 sec

Number of cycles: 30

PCR-2: Optimization with different temperature programme

The same sample mixtures were prepared as in PCR-1 but were labelled as PCR-2-1, PCR-2-2, and PCR-2-3. This is because PCR was carried out using a temperature programme which is different from that of PCR-1.

Denaturing: 91°C, 1 min

Annealing: 60°C, 1 min

Extension: 65°C, 3 min + 10 sec

Number of cycles: 30

In this programme, each cycle was edited separately to allow 10 additional seconds to the extension period. Hence, cycle 1 was edited for 3 minutes at 65°C for extension, cycle 2 for 3 minutes 10 seconds, cycle 3 for 3 minutes 20 seconds, etc, for up to 30 cycles.

PCR-3: Optimization of PCR using two different $MgCl_2$ concentrations

In this experiment, a different Mg(II) concentration, 0.78 mM was used in the final PCR reaction mixture instead of 7.8 mM in the previous PCR and 200 ng of each primer was used for each reaction.

Sample	DNA Template	Primers (@ 200ng)	$MgCl_2$	Water
PCR-3-1	Nil	102&103	7.8 mM	36 µl
PCR-3-2	CPP-1: 1 µl (161 ng)	102&103	7.8 mM	35 µl
PCR-3-3	WPC-1: 3 µl (198 ng)	102&103	7.8 mM	33 µl
PCR-3-4	Nil	103&104	0.78 mM	36 µl
PCR-3-5	CPP-1: 1 µl (161 ng)	103&104	0.78 mM	35 µl
PCR-3-6	WPC-1: 3 µl (198 ng)	103&104	0.78 mM	33 µl

Taq DNA polymerase: 2.5 units

Temperature programme:

Denaturing: 95°C, 2.5 min

Annealing: 60°C, 2.5 min

Extension: 70°C, 4 min

Number of cycles: 30

PCR-4: PCR with different programme and primers 103 and 104

Sample	DNA Template	Primers	Water
PCR-4-1	Nil	2 μl (200 ng)	36 μl
PCR-4-2	CPP-1: 4 μl (560 ng)	2 μl (200 ng)	32 μl
PCR-4-3	WPC-1: 8 μl (528 ng)	2 μl (200 ng)	28 μl
PCR-4-4	CPP-3: 8 μl (560 ng)	2 μl (200 ng)	28 μl

Taq DNA polymerase: 2.5 units

Temperature programme:

Denaturing: 93°C, 2 min

Annealing: 60°C, 2 min

Extension: 65°C 3, 4, 5, 6, 7, or 8 min

Using the same TempCycler, six separate profiles were edited, each with the same set of parameters except that the period of the extension increased from 3 to 8 minutes. Each profile was then run for 6 cycles, i.e. the first 6 cycles had an extension period of 3 minutes and the next 6 cycles for 4 minutes, etc. In this programme, a total of 36 cycles were run.

PCR-5: PCR with primers 101 and 102

Oligonucleotides 101 and 102 were used as primers, 80 ng each, otherwise the composition of WPC-1 and CPP-3 samples, the temperature programme and the number of cycles were the same as in PCR-4.

PCR-6: Optimization using different amounts of templates

Sample	DNA Template	Primers	Water
PCR-6-1	Nil	10 µl (400 ng)	20 µl
PCR-6-2	CPP-2: 2 µl (140 ng)	10 µl (400 ng)	18 µl
PCR-6-3	CPP-2: 4 µl (280 ng)	10 µl (400 ng)	16 µl
PCR-6-4	WPC-1: 2 µl (130 ng)	10 µl (400 ng)	18 µl
PCR-6-5	WPC-1: 4 µl (260 µl)	10 µl (400 ng)	16 µl

Taq DNA polymerase: 2.5 units

The temperature programme was same as in PCR-4.

PCR-7: Optimization using a different PCR machine

Sample	DNA Template	Primers	Water
PCR-7-1	Nil	10 µl (400 ng)	20 µl
PCR-7-2	CPP-2: 2 µl (140 ng)	10 µl (400 ng)	18 µl
PCR-7-3	WPC-1: 2 µl (130 ng)	10 µl (400 ng)	18 µl
PCR-7-4	CPP-2: 4 µl (280 ng)	5 µl (200 ng)	16 µl
PCR-7-5	WPC-1: 4 µl (260 ng)	5 µl (200 ng)	16 µl
PCR-7-6	CPP-2: 8 µl (560 ng)	10 µl (400 ng)	12 µl
PCR-7-7	WPC-1: 8 µl (520 ng)	10 µl (400 ng)	12 µl

Taq DNA polymerase: 2.5 units

The temperature programme was same as in PCR-2.

3.3.6 Purification of PCR Products

A commercially available GENECLEAN Kit was purchased from BIO 101 (La Jolla, CA, USA) and it contained the following:

NaI: 6M sodium iodide solution

TBE Modifier: a proprietary mixture of concentrated salts.

NEW Concentrate: for making the NEW wash, (NaCl/ethanol/water).

GLASSMILK: a suspension of silica matrix in water.

Twenty microlitres of PCR product was electrophoresed in 1.4% (w/v) agarose gel in 1 X TAE buffer at 50 milliamperes for 1 hour, the appropriate DNA band was excised under uv. The gel was weighed, put into an eppendorf tube, and added with 3 volumes of NaI solution and incubated at 52°C for several minutes until the gel was dissolved. Glassmilk, 5 μl, was added and the mixture incubated on ice for 5 minutes and vortexed gently at 1-minute intervals. The suspension was then centrifuged at 13000 X g for 5 minutes. The supernatant was discarded and the pellet (DNA) washed by 200 μl NEW wash thrice. The DNA adsorbed on the Glassmilk was eluted with water. Firstly, 6 μl of water was added to resuspend the pellet and incubated at 52°C for 3 minutes. The supernatant was collected after centrifugation at 13000 X g for 1 minute. This process was repeated with another 6 μl of water. The supernatants were combined. DNA content was determined by uv spectrophotometry and polyacrylamide gel electrophoresis.

At times when the GENECLEAN procedure failed to work as expected, i.e., yield of DNA was poor, the following rescue procedure was carried out. Twenty microlitres of the PCR product was electrophoresed on 1.4% (w/v) agarose gel in 1 X TAE buffer. The gel containing the DNA band was excised under uv. Sodium iodide solution, 370 μl, was added incubated at 52°C for 8 minutes. The pH was adjusted to 6 after adding dilute acetic acid. Twenty microlitres of this solution was kept aside, and 5 μl Glassmilk suspension was added to the rest of NaI/DNA mixture on ice. The suspension was mixed gently every minute for 5 minutes. The pellet obtained after centrifugation at 13000 X g for 10 seconds was washed with the NEW wash thrice, while the supernatant was kept aside. The NEW washes were combined. DNA adsorbed on the pellet was eluted by incubation with 10 μl water at 52°C for 5 minutes. The eluate was collected after centrifugation at 13000 X g for 1 minute.

Agarose Gel Extraction with Glasswool Exclusion

PCR product (20 μl) was electrophoresed in 1.4% (w/v) agarose gel in 1 X TAE buffer at 50 milliamperes for 1 hour. The appropriate DNA band in the gel was excised under uv and cut into 5 equal portions. Each gel slice was placed into a 0.5 ml small eppendorf tube containing and 100 μl TE pH 8.0 (Fig.4). A hole had been punched from the bottom of the tube which was stuffed with small amount of siliconized glasswool and placed inside a 1.5 ml eppendorf tube. The gel was then

allowed to dissolve. The tube assembly was centrifuged at 13000 X g at 4°C for 5 minutes. Upon centrifugation, eluate coming out from the hole of the small tube entered to the 1.5 ml tube. To the eluate was added 3 M sodium acetate to a final concentration of 0.3 M and 2.5 volumes of absolute ethanol. DNA was precipitated by incubation at -70°C for 1 hour and pelleted by centrifugation at 13000 X g for 5 minutes. After washing with 70% ethanol, the DNA pellet was air dried and dissolved in 20 µl TE buffer.

Polyacrylamide Gel Extraction

The PCR product (20 µl) was electrophoresed in 5 % (w/v) polyacrylamide gel at 60 milliamperes for 30 minutes. The gel was treated with ethidium bromide and DNA viewed under uv. The DNA band in the appropriate position was excised and put into eppendorf tubes. A 100 µl aliquot of 2 M ammonium acetate and 5 µl 1% triton X-100 were added. The mixture was incubated overnight at 37°C with constant shaking. Equal volume of n-butanol was then added and the mixture vortexed for 5 minutes and centrifuged at 13000 X g for 1 minute. The upper organic layer was aspirated off. To the lower aqueous layer was added 60 µl 5 M ammonium acetate and 3 volumes of absolute ethanol. The mixture was incubated at -20°C for 1 hour. DNA pellet was collected by centrifugation at 13000 X g for 30 minutes, then washed with 500 µl 70% ethanol. The DNA sample was air dried, and dissolved in 10 µl TE buffer.

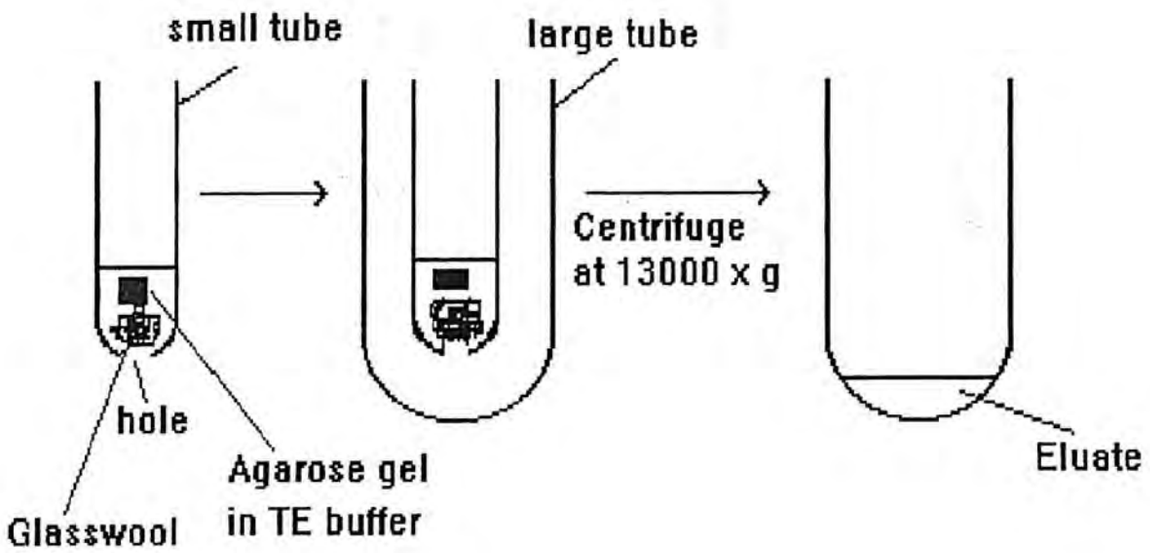

Fig. 4 Purification of PCR product by agarose gel extraction and glasswool exclusion.

3.2.10 **Sequencing**

The composition of reagents and solutions used in sequencing reactions is detailed as follows:

ddNTP Mixes

T Mix: 80 μM dTTP, dCTP, and dGTP; 8 μM ddTTP;
50 mM NaCl; 10% DMSO.

C Mix: 80 μM dTTP, dCTP, and dGTP; 8 μM ddCTP;
50 mM NaCl; 10% DMSO.

G Mix: 80 μM dTTP and dCTP; 8 μM dGTP and ddGTP;
50 mM NaCl; 10 % DMSO.

A Mix: 80 μM dTTP, dCTP, and dGTP; 0.08 μM ddATP;
50 μM NaCl; 10 % DMSO.

Enzyme Labelling Mix: 2 μl ^{35}S-ATP
0.5 μl sequenase (Klenow Fragment)
2 μl 100 mM DTT
1 μl DMSO
3.5 μl Tris $EDTA_{0.1}$ pH 8

Total volume 9 μl, for 2 reaction.

Annealing Mix: 0.5 μl 100ng/μl primer
5 μl 5 X sequenase buffer
2 μl DMSO
3.5 μl Tris $EDTA_{0.1}$ pH 8.0

Chase Solution: 2 μl 15mM dNTPs
12 μl DMSO
106 μl H_2O

Total volume 11 μl, for 2 reactions.

Stop Solution: 98% formamide

10 mM EDTA

0.3% (w/v) xylene cyanol

0.3% (w/v) bromphenol blue

A commercial S^{35}Sequencing Kit purchased from Pharmacia (Uppsala, Sweden) was also used, the kit contained:
'A' mix: ddATP in solution with dATP, dCTP, dGTP and dTTP
'C' mix: ddCTP in solution with dATP, dCTP, dGTP and dTTP
'G' mix: ddGTP in solution with dATP, dCTP, dGTP and dTTP
'T' mix: ddTTP in solution with dATP, dCTP, dGTP and dTTP
FPLCpure Klenow Fragment, sequencing buffer, chase solution and stop solution. The stop solution was a formamide solution containing 10mM EDTA, 0.3% (w/v) xylene cyanol and 0.3% (w/v) bromphenol blue.

The gradient of 6% (w/v) denaturing PAGE gel was prepared by the following:

The light acrylamide solution (A) contained: 0.5 X TBE/6% acrylamide/ 7.67 M urea. The heavy acrylamide solution (B) contained 2.5 X TBE/ 6% (w/v) acrylamide/ 7.67 M urea/ 10% (w/v) sucrose. Both solutions were filtered and degassed. One millilitres of both acrylamide solutions were added with 1 µl 25% ammonium persulphate and 1 µ TEMED. Twelve millilitres of light solution was pipetted into a 25 ml pipette. This was followed by drawing 12 ml of the heavy

solution into the same pipette. The gradient interface was mixed by a few dislodged bubbles. Contents of this pipette was cast down into the space of the sandwich glasses used for gel setting. Another 50 ml of light solution was layered on top. A comb was placed into the gel to form slots for sample loading.

Gel Drying

The gel after electrophoresis was fixed in acetic acid: methanol: water (1:1:8 v/v/v) for 15 minutes before being transferred to a sheet of Whatman 3 mm paper. It was dried on a Gel Dryer (BioRad, Richmond, CA, USA).

Autoradiography

After the gel was completely dried, it was put into a X-ray film cassette and autoradiographed with Du Pont Cronex intensifying film by exposure for 16-24 hours at room temperature.

Sequencing Protocol-1

Steps involved in sequencing are described as follows:

1. Four microlitres of a purified DNA as template was added to 5 μl annealing mix. After incubation at 95°C for 3 minutes, the mixture was snapped cool in liquid N_2 in which it was kept for a further 5 minutes. The sample was allowed to thaw out at room temperature for

10 minutes.

2. After adding 4 μl of enzyme labelling mix to the sample, the reaction mixture was divided into 4 X 2.5 μl portions. Two microlitres of each A, C, G, and T mix was added to each of the 4 portions. The samples were then incubated at 37°C for 5 minutes.
3. Two microlitres of chase solution was added to each reaction mixture, which was then incubated at 37°C for another 5 minutes. This is followed by the addition of 3 μl stop solution.
4. Each A, C, G, and T reaction mixture was then heated to 75-80°C for 2 minutes prior to electrophoresis on 6% denaturing polyacrylamide gel.

Sequencing Protocol-2

This protocol utilized most of the solutions in the ^{35}Sequencing kit supplied by Pharmacia and the steps involved are outlined as follows:

1. Double-stranded DNA was denatured by incubating a mixture of 2 μl DNA solution, 6 μl water and 2 μl 2 M NaOH at room temperature for 10 minutes.
2. To the mixture was added 3 μl 3 M sodium acetate, pH 4.5, 7 μl distilled H_2O and 60 μl absolute ethanol. It was then placed on ice for 5 minutes and transferred to liquid N_2 for 15 minutes. After thawing the sample was centrifuged. The DNA pellet obtained was washed with 500 μl ice-cold 70% ethanol, air-dried and redissolved

in 1 μl H_2O.

3. Sequencing buffer, 1.5 μl, and 7.5 μl of 100 ng / μl oligonucleotide 101 as primer were added, and the sample incubated at 37°C for 20 minutes to allow annealing of the primer onto the template.

4. An enzyme/label/primer/template (ELPT) mixture was prepared by adding 3 μl of FPLCpure Klenow Fragment and 2 μl (20 μCi) of [α-^{35}S]dATPαS (600 Ci/mmol) to the annealed primer/template from step 3.

5. Four sequencing reactions were set up 3 μl of each A, C, G, and T mixes was put into each labelled tube, to which 3 μl ELPT was added. Reaction was initiated by centrifugation of the samples at 13000 X g for 2 seconds, and proceeded at room temperature for 15 minutes.

6. Towards the end of the incubation period, 1 μl of chase solution was placed on the wall of each tube, and was allowed to mix with the sample by centrifugation for 2 seconds. The sample mixtures stood at room temperature for 15 minutes.

7. Towards the end of this second period of incubation, 3 μl of stop solution was then placed on the wall of each tube. Mixing was initiated by centrifugation to stop the sequencing reaction.

8. The samples were heated at 95°C for 3 minutes prior to electrophoresis on denaturing polyacrylamide gel.

3.2.11 Cloning

The purified PCR product was used as DNA insert, the staggered ends were trimmed with T_4 DNA polymerase in the following reaction mixture:

DNA samples	1 μl
T4 DNA polymerase	0.5 μl
dNTP 10mM	0.5 μl
T4 polymerase buffer	1 μl
H_2O	7 μl

This mixture was incubated at 30°C for 45 minutes and then at 75°C for 10 minutes.

The vector M13 (50 μl) was digested with 2 μl of Sma I restriction endonuclease, 6 μl of 10 X buffer, and 2 μl of H_2O, then incubated at 25°C for 2 hours. A second digestion was done by a further addition of 2 μl Sma I, 1 μl 10 X buffer and 7 μl H_2O and incubation at 25°C overnight. The treated vector M13 (70 μl) was dephosphorylated by adding 1 μl of calf intestinal alkaline phosphatase, 8 μl buffer and 1 μl H_2O and incubating at 37°C for 30 minutes, then at 56°C for 30 minutes. An additional 1 μl alkaline phosphatase was added and the reaction mixture was incubated at 37°C for 45 minutes, then at 56°C for 45 minutes. The M13 was heated at 75°C for 10 minutes, followed by GENECLEAN purification.

A ligation cocktail was prepared:

Sample	1	2	3	4	5
Vector M13 (μl)	1	1	1	1	1
Insert DNA (μl)	1	4	1	4	-
T4 ligase buffer (μl)	1	1	1	1	1
T4 ligase (μl)	1	1	1	1	1
Water (μl)	6	3	6	3	7

Sample 5 was used as control for vector.

Competent cells were prepared as the following:

1. TG1, a strain of Escherichia coli for transformation of vector M13 was grown at 37°C with shaking overnight. A well isolated colony of TG1 was inoculated in 5 ml of LB broth.
2. After overnight incubation, 0.1 ml of TG1 was inoculated to 21 ml of LB broth and incubated at 37°C for 3 hours with constant shaking. The culture was then cooled on ice for 15 minutes. The cell pellet obtained by centrifugation was resuspended in 10 ml of ice cold 100 mM $CaCl_2$. After 20 minute incubation on ice, the suspension was centrifuged and the cell pellet resuspended in 2 ml ice cold 100 mM $CaCl_2$. To 5 μl of each ligation sample, 0.2 ml cells was added for transformation.

3. Each pre-cooled polypropylene tube was added 200 μl competent cells, and 5 μl DNA cocktail. The sample mixture was then put into molten top agar (3 ml for each tube) and mixed with 40 μl 2.5% IPTG and 40 μl 2 % X-Gal.
4. The agar mixture was then poured onto a LB plate and allowed to set for a few minutes.
5. The plate was incubated at 37°C overnight.

4. RESULTS

4.1 DNA extraction

Normal subject, CPP

Several attempts were made to extract and purify genomic DNA from 10 ml fresh blood of a normal subject (CPP). The blood collected was carefully centrifuged to obtain a clear buffy coat layer which contained predominantly leucocytes and lymphocytes. Nucleic acids were prepared from this buffy coat fraction.

The amount of genomic DNA obtained in the first attempt was quite satisfactory. Its OD 260/ OD 280 ratio of was 1.77 (Table 1) and its uv absorption spectrum from 250 nm to 300 nm shown in (Fig 5). The absorption data suggested that this DNA sample contained very little contamination of protein. It was labelled CPP-1.

A modification in extraction condition was made in the second attempt with the view to improve the DNA yield. Lysing buffer 2 was used. It contained 5 mM EDTA instead of 1 mM EDTA in lysing buffer 1 as used in the first attempt. The DNA sample obtained was labelled CPP-2. It had a low OD 260/OD 280 ratio of 1.39, which suggested the presence of considerable amount of protein. The uv spectrum (Figure 6) confirmed this, i.e. high absorption in the 280 nm region.

Table 1. Quantitation of DNA samples. The DNA yield was estimated by absorption at 260 nm.

SAMPLE	CPP-1	CPP-2	CPP-3	WPC-1	PCR-1-1	PCR-1-2
DNA YIELD (ug)	48.50	52.00	20.90	19.77	3.88	3.03
OD260/OD280	1.77	1.39	1.87	1.83	2.43	3.56

Although the DNA yield, on the basis of absorption at 260 nm, was similar to that of the first attempt, the DNA was not as pure as the first sample CPP-1. Sample CPP-2 was therefore treated with phenol and phenol/chloroform again. The product had an OD 260/OD 280 ratio of 1.87 and renamed as CPP-3. It was probably due to these further purification steps, the yield was low. However, its uv spectrum showed that there was less interference in the 280 nm region, after the further treatment with phenol and phenol/chloroform (1:1).

A satisfactory DNA preparation from the patient was, on the other hand, obtained using the same procedure (Table 1 and Figure 3). It was labelled as WPC-1 and was subsequently used for PCR.

4.2 **Calibration of the Coy TempCycler**

Since preliminary PCR attempts carried out on the TempCycler (Coy) were not successful, it was suspected that the assigned temperature on the machine has not been reached. The thermocycler was hence re-calibrated using a 1100 thestoterm thermometer. The temperature probe was inserted directly into a PCR tube containing water and paraffin oil. The tube was placed in the thermocycler. Three cycles of temperature programme were set:

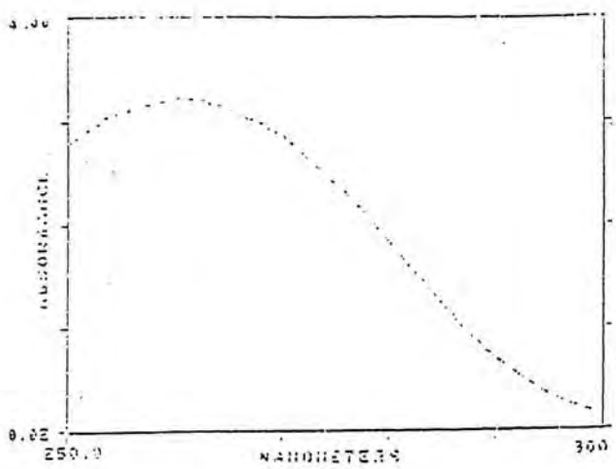

Fig. 5 Absorption spectrum of CPP-1

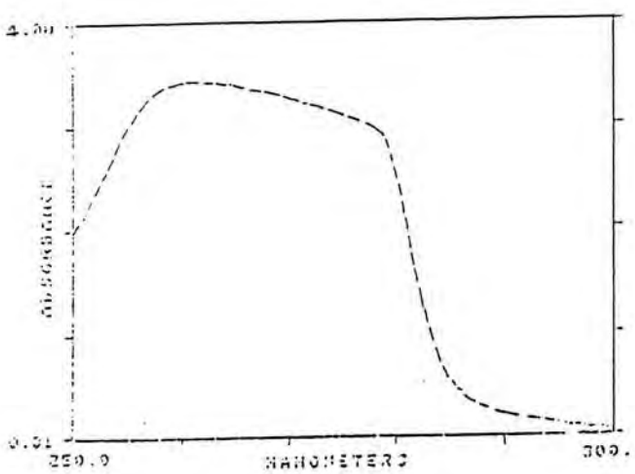

Fig. 6 Absorption spectrum of CPP-2

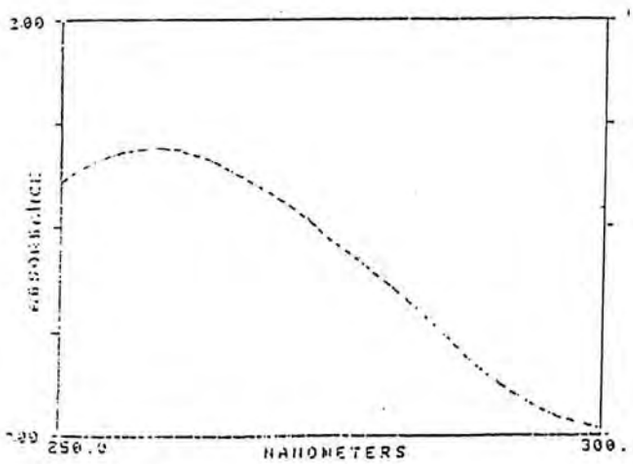

Fig. 7 Absorption spectrum of CPP-3

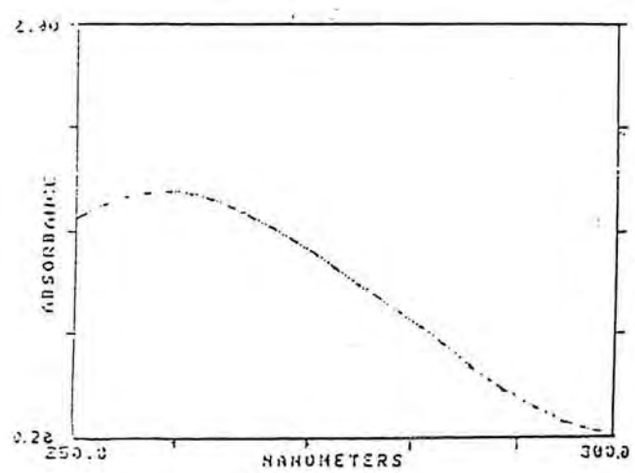

Fig 8. Absorption spectrum of WPC-1

Denaturation:	91°C,	3 min
Annealing:	60°C,	3 min
Extension:	65°C,	3 min

The temperature was checked at 1-minute intervals. From the results of Table 2, the actual temperature at the set point for denaturation (91°C) was about 75°C, it took one minute to reach 89°C and another two minutes to 91°C. The next set point was at 60°C, however, the actual temperature was still at 81°C, when the machine read 60°C two more minutes were needed to reach the actual set temperature of 60°C. The thermocycler was therefore re-calibrated for subsequent temperature programming.

4.3 Optimization of PCR

4.3.1 PCR-1: Photo 1

Lane 1 and 10: 2 μl Hae III Digest marker (total 100 ng)

Lane 2 and 3: 2 μl loading dye

Lane 4 and 9: 5 μl PCR-1-1

Lane 5: 5 μl PCR-1-2

Lane 6: 10 μl PCR-1-2

Lane 7: 5 μl PCR-1-3

Lane 8: 10 μl PCR-1-3

Results:

1. As the markers appeared smeared, the molecular weight of PCR products could not be reliably estimated.

Table 2 Calibration of the Coy Tempcycler. The actual temperatures were recorded by an electronic 1100 thestoterm thermometer.

CYCLE	SET TEMPERATURE °C	ACTUAL TEMPERATURE AT SET POINT °C	ACTUAL TEMPERATURE AFTER 1 MIN. °C	ACTUAL TEMPERATURE AFTER 2 MIN. °C	ACTUAL TEMPERATURE AFTER 3 MIN. °C
1	91	75.1	89.2	90.6	90.8
	60	81.2	61.6	60.1	59.8
	65	59.8	64.2	64.6	64.7
2	91	75.7	89.2	90.8	90.9
	60	82.1	62.0	60.2	59.9
	65	81.0	61.9	60.3	60.0
3	91	75.7	89.4	90.8	90.9
	60	82.1	62.0	60.2	59.9
	65	60.1	64.3	64.8	64.9

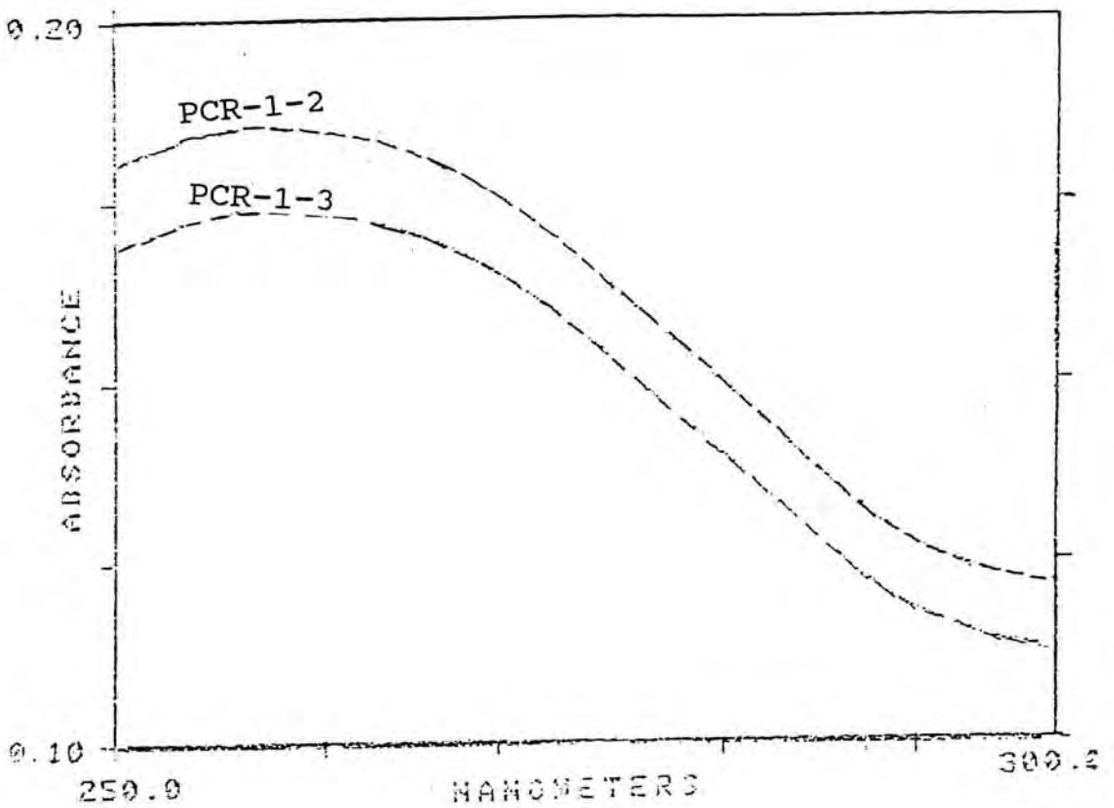

Fig. 9 Absorption spectrum of PCR-1-2 & PCR-1-3.

band was detected in PCR-3-4 and PCR-3-5. The yield of amplification not improved using less $MgCl_2$ in this protocol.

4.3.4 PCR-4: Photo 2

Lane 1: loading buffer

Lane 2: 3 μl PATX marker

Lane 3: 2 μl PCR-4-2

Lane 4: 2 μl PCR-4-2

Lane 5: 2 μl PCR-4-3

Lane 6: 2 μl PCR-4-4

Lane 7: 2 μl PCR-4-1

Results:

1. Slight degradation on the molecular weight marker was shown on lane 2, separated DNA fragments had the molecular weight of 1300, 517, 396, 298, 221, 220, 150, 145, and 75 base pairs.
2. Two products amplified from template CPP-1 were shown on lanes 3 and 4, similar yield were obtained at the position appropriate on the gel for 332 bp.
3. In lane 5, the yield of PCR-4-3 prepared from WPC-1 template was slightly less than other 3 lanes, however, less nonspecific smearing was observed.
4. Other product amplified from CPP-3 template (PCR-4-4) showed similar yield.
5. Results obtained in this experiment were, deemed satisfactory.

4.3.5 PCR-5: Photo 3

Lane 1: loading buffer

Lane 2: 3 μl Hae III Digest pBR322 DNA marker (total 420 ng)

Lane 3: 1 μl PCR-5-1

Lane 4 and 5: 1 μl PCR-5-2

Lane 6, 7, and 8: 1 μl PCR-5-3

Results:

1. Separated DNA fragments in Hae III cut pBR 322 DNA: 587, 540, 504, 458, 434, 267, 234, 213, 192, 184, 124, 123, 104, 89, 80, 64, 57, 51, 21, 18, and 11 base pairs was used as marker. For the low molecular weight fragments, separation was not so obvious. The PCR product was situated at the expected position of 334 bp, in between the 434 and 267 bp markers.
2. Less amount of primer was used in this experiment, but significant amplification was still obtained.
3. In lanes 4 and 5, slight nonspecific background was found at lower part of PCR product which might be due to the quality of template.

4.3.6 PCR-6: Photo 4

Lane 1: loading buffer

Lane 2: 2 μl Hae III Digest pBR322 DNA marker (total 280 ng)

Lane 3 and 8: 2 μl PCR-6-1

Lane 4: 2 μl PCR-6-2

Lane 5: 2 μl PCR-6-3

Lane 6: 2 μl PCR-6-4

Lane 7: 2 μl PCR-6-5

Results:

1. Only half of the amount of template was used in PCR-6-2 (lane 4) comparing with PCR-6-3 (lane 5), but slightly higher yield was obtained. The background smearing was slightly stronger in PCR-6-2.
2. Comparing to PCR-5, PCR-6-3, PCR-6-5 had 5 fold increase in amount of primer, and 2-fold decrease in template, whilst PCR-6-2 and PCR-6-4 5-fold increase in primer and 4-fold decrease in template. No significant differences in terms of the yield were observed.
3. Less nonspecific smearing was found in lanes 6 and 7, which were PCR products obtained from the haemophiliac patient. This was consistent with results of PCR-5.

4.3.7 PCR-7: Photo 5

Lane 1: loading dye

Lane 2: 2 μl Hae III Digest pBR322 DNA marker (total 280 ng)

Lane 3 and 10: 2 μl PCR-7-1

Lane 4: 2 μl PCR-7-2

Lane 5: 2 μl PCR-7-3

Lane 6: 2 μl PCR-7-4

Lane 7: 2 μl PCR-7-5

Lane 8: 2 μl PCR-7-6

Lane 9: 2 μl PCR-7-7

Results:

1. The amounts of primer and template in PCR-7-2 and PCR-7-3 were same as PCR-6-2 and PCR-6-4 respectively. Better yield was obtained in this protocol which might be due to faster ramp time for the set point of temperature programme minimizing the nonspecific annealing.
2. Both PCR-7-6 and PCR-7-7 had better yields and less nonspecific background compared with other pairs of samples.
3. New batches of Taq DNA polymerase and amplification buffer were used. No contamination was found in the water control (lanes 3 and 10).

4.4 Purification of PCR Products

GENECLEAN II Kit BIO 101

4.4.1 GC-1: Photo 6

Lane 1: loading dye

Lane 2: 3 μl PATX $Hinf_1$ marker

Lane 3: 2 μl Hae III Digest ϕX-174-RF DNA marker (total 100 ng)

Lane 4: 1 μl gene cleaned PCR-4-2

Lane 5: 1 μl gene cleaned PCR-4-3

Lane 6: 5 μl gene cleaned PCR-4-2

Lane 7: 5 μl gene cleaned PCR-4-3

The procedure recommended in the kit was followed, but the yield was very low. No detectable DNA fragment was found in 1 μl eluent on 5% (w/v) PAGE. Eluent with 5 μl DNA fragment of PCR-4-2 and PCR-4-3 were degraded (lanes 6 and 7). More than 80% of the original DNA sample were lost.

4.4.2 GC-2

In this attempt very strict precaution had been taken: the Glassmilk suspension was thoroughly mixed, samples kept on ice throughout the purification procedure and so on. But no satisfactory improvement was made. The yield was still very low.

A trouble-shooting procedure was taken, and the products again analysed on PAGE. Faint bands were found near the region of PCR product, suggesting that the first and second elution were not complete. Another faint band was appeared in the eluent eluted from the Glassmilk, showing weak adsorption.

4.4.3 **GC-3: Photo 7**

Lane 1: 2 μl Hae III Digest pBR322 DNA marker (total 280 ng)

Lane 2: 1 μl PCR-6-2, no GENECLEAN treatment

Lane 3: 1 μl PCR-6-2 purified by GENECLEAN

Lane 4: 1 μl PCR-6-3, no GENECLEAN treatment

Lane 5: 1 μl PCR-6-3 purified by PAGE

Lane 6: 1 μl PCR-6-4, no GENECLEAN treatment

Lane 7: 1 μl PCR-6-4 purified by GENECLEAN

Lane 8: 1 μl PCR-6-5, no GENECLEAN treatment

Lane 9: 1 μl PCR-6-5 purified by PAGE

Results:

1. Only a faint band was shown on lanes 3 and 6.
2. Samples PCR-6-2, PCR-6-5, PCR-6-3 in lanes 2, 6, and 8 without treatment were shown to have nonspecific background.
3. In lane 4, very faint band was observed which might be due to inappropriate loading of sample.
4. Purification of PCR-6-2 and PCR-6-4 with GENECLEAN method showed very low recovery.

4.4.4 PAGE-1: Photo 7

The same photograph was used as in GC-3. In lanes 5 and 9, more than 90% of PCR product were yielded from PAGE purification. Very clean bands were observed suggesting that this purification method was significantly better than the other two methods discussed previously.

4.4.5 PAGE-2: Photo 8

Lane 1: 2 μl Hae III Digest pBR322 DNA marker (total 280 ng)

Lane 2: 1 μl PCR-7-2 before gene purification

Lane 3: 1 μl PCR-7-2 purified by PAGE

Lane 4: 1 μl PCR-7-3 before purification

Lane 5: 1 μl PCR-7-3 purified by PAGE

Lane 6: 1 μl PCR-7-4 before purification

Lane 7: 1 μl PCR-7-4 purified by PAGE

Lane 8: 1 μl PCR-7-5 before purification

Lane 9: 1 μl PCR-7-5 purified by PAGE

Results:

1. In lanes 2, 4, 6, and 8, samples were shown to have very low nonspecific background on the edges of wells and some fluorescent smearing on the lane.
2. All purified products from lanes 3, 5, 7, and 9 gave satisfactory yield of more than 90% recovery.
3. Purification of PCR products could be extracted from

PAGE for subsequent experiments.

4.4.6 Agarose Gel Extraction with Glasswool Exclusion: Photo 9

Lane 1: loading dye

Lane 2: 1 μl Hae III Digest pBR322 DNA marker (total 140 ng)

Lane 3: 1 μl out of 20 μl PCR product of PCR-4-3

Result:

1. The markers in lane 2 were somehow lost.
2. Purification on agarose gel with glasswool exclusion shown on lane 3 was better than samples in GC-1 and GC-3 lanes 3 and 7 by GENECLEAN, however, degraded bands appeared in this method.

4.5 Direct Sequencing of PCR product

DS-1

Self-prepared reagents were used, heat denaturation of DNA template followed by snap cool on liquid N_2 was employed. However, no detectable bands were observed.

DS-2

Procedures and reagents of the ^{35}Sequencing Kit obtained from Pharmacia was followed. However, no detectable bands were observed.

DS-3

Procedures and reagents of ^{35}Sequencing Kit were used to repeat the direct sequencing again. Some nonspecific signals were appeared on the film, but more than one bands were appeared at the same level.

DS-4

The same procedures and reagents were adopted as described in DS-3. However, higher amounts of template, enzyme and primer were used. Improved signals appeared on some level. Nevertheless, the intensity was too weak to enable the actual sequences to be read. There were also non-specific signals.

4.6 Cloning

Cloning-1

Only white plaques were observed on the LB agar plate.

Cloning-2

No plaques were formed.

Cloning-3

The vector M13 was purified by GENECLEAN. The molecular weight of M13 is about 700 bp, however, smears of bands were observed on PAGE. Only a few white plaques and much more blue plaques appeared, the quality of the GENECLEAN was again doubtful.

5. DISCUSSION

5.1 DNA Extraction

Different protocols have been used for DNA preparation from blood samples. Basically, most methods employed in the present study involve protein digestion by Proteinase K, RNA removal by RNase and multiple solvent extraction. They differ mainly in the details of reaction conditions. In one of the attempts, samples were first mixed vigorously by vortexing for a few minutes, then centrifuged at room temperature, 8000 X g for 20 minutes. After this extraction, samples were found to be warm. I did not obtain good DNA. In separate attempt, the tubes were inverted gently and repeatedly for 3 minutes to facilitate mixing. These samples were centrifuged at 4°C, 4000 X g, for 10 minutes instead of 20 minutes. By taking such precautions, it appeared to be of some help in obtaining good DNA. The procedure modified by Blin and Stafford [31] which recommended to use a wide-bore pipette (0.3-cm-diameter orifice) to transfer the aqueous phase to a microtube for phenol/phenol chloroform extraction. their recommendation was followed.

5.2 Polymerase Chain Reaction

To establish a reliable PCR, I found that optimization of the PCR conditions is necessary. Important factors in achieving this goal include quality of DNA as template,

concentration of primers, incubation time and temperature during the annealing and extension steps. Although initial PCR conditions and temperature parameters during the first part of this study were based on an established methodology [28], results obtained were not entirely satisfactory. Several attempts to optimize PCR were still necessary in order to obtain a protocol capable of producing positive and reproducible amplification results. Once a workable protocol was developed, details of conditions must be adhered strictly. I found that the stringency of conditions was extremely important. Several technical precautions have to be taken. There must be absolute avoidance of carry-over of reagents. The Taq polymerase enzyme must be kept 'clean'. New pipette tips had been used in each aliquoting. Gloves and clean laboratory coats should be worn during the experiment, reagents were aliquoted and frozen for each experiment.

Concentration of magnesium ion has been shown to have significant effect on the amplification process. It may affect primer annealing, strand dissociation temperatures of both template and PCR product, product specificity, formation of primer-dimer artifacts, and enzyme activity and fidelity [32]. The presence of chelating agents such as EDTA or negatively charged ionic groups such as phosphates may affect the equilibration of magnesium ionization. However, I found no significant difference by changing the $MgCl_2$

concentration of 10 fold difference in $MgCl_2$. Meanwhile, the amount of purified DNA used as template in my PCR was quite high. As much as 550 ng of DNA was needed to give good amplification results. Probably, the DNA preparations were not pure enough.

Most of the published methods of PCR work on the FIX gene were carried out using a Cetus Thermocycler. The specification of this instrument, particularly the speed of the temperature changes were different from the Coy TempCycler used in the present study. I Therefore needed to make some adjustments had to be made on the PCR temperature programme with different thermocyclers. I also found that the display temperature in my instrument was slightly different from that of the actual temperature. It was for this reason, I re-calibrated the Coy TempCycler with an electronic thermometer. Hence, the thermocycler was found to be stable and consistent with performance, which were vital for development of PCR programmes.

5.3 Purification of PCR Products

To purify the amplified DNA material after PCR, I used a GENECLEAN II kit. It has been used widely by many workers in different laboratories and is shown to be convenient, rapid, simple and reliable. It has been claimed that more than 80% of the purified DNA fragment can be recovered, and

impurities such as RNAs, proteins, ethidium bromide, residual phenol, chloroform and unincorporated nucleotide and primers are removed effectively. However, I repeatedly obtained less than 50% yield. Sometimes over 90% of starting materials were lost. A procedure for trouble-shooting was tried. The result showed that this excised DNA fragment die not bind to the Glassmilk effectively. A second GENECLEAN II kit was obtained from the supplier. But again poor yield and inconsistent results were obtained. It is possible that my PCR product was too small, 332 bp, for effective adsorption onto the Glassmilk. The GENECLEAN manual did specify that a 80% yield should be obtained for DNA fragment larger than 500 bp. GENECLEAN of other PCR products more than 300 bp in length have produced more than 50% yield in this laboratory [33]. The introduction of agarose gel extraction with glasswool as exclusion medium led to a better yield. However, slight degradation of the DNA fragment was found in repeated trials. This procedure was simple to set up, but the time required for preparation and elution was long. Extraction of DNA from the polyacrylamide gel after electrophoresis was found to be an easy-to-handle method. In repeated trials recoveries of DNA were consistently higher than 90%. The drawback is that this is not an exhaustive purification procedure. The DNA band on the gel which was excised for DNA extraction might obtain other materials adhering to the DNA product and electrophoresed to the same position. However, this was the

only purification procedure which gave consistent good recoveries. I used this method to prepare DNA for subsequent DNA sequencing experiment.

5.4 Sequencing

Sequencing experiments utilizing a commonly supplied reagent kit were not successful. The DNA extracted from the polyacrylamide gel was used directly for sequencing after alkalination. Although the sequencing procedures were followed strictly and repeated several times. I always obtained very dark bands at the top of the gel, with faint bands at the lower part. Moreover, there are bands in more than one track at the same level. Since the dXTP/ddXTP ratios of this ^{35}Sequencing kit were not provided, I was unable to manipulate their concentrations to improve the sequencing reactions. I thus switched to a protocol based on a published method [28] and made up all reagents by myself. Unfortunately the reactions again did not work. The failure of these experiments were probably due to the poor quality of the DNA template, which was not sufficiently pure.

5.5 Cloning

I intended to clone the PCR DNA of FIX promoter into M13 phage as this could produce large amount of DNA which could be purified for sequencing. To facilitate cloning of the amplified DNA into M13, selected restriction sites can be

incorporated at the 5′ ends of the amplification primers. For the first attempt for cloning, only a few white plaques were observed on LB agar plate which indicated that the vector-insert ligation had failed to take place. The vector could have self-ligated prior to phosphatase treatment. One of the most likely causes is that the molar ratio of vector DNA to insert DNA was not desirable for ligation to proceed. This might be rectified by repeating the ligations at a range of vector-insert ratios. Another likely cause is that the insert material contained ligase inhibitors or has unsuitable ends.

The second cloning experiment was also unsuccessful. There was no formation of plaques, indicating a very low transfection efficiency, the competent cells might be too old to be used. In the third cloning attempt new competent cells were prepared. But still only very few white plaques appeared, other plaques were blue. This indicated that either the ligase has low activity, or the restriction enzyme had failed to digest the vector, leaving closed circular material, i.e. re-ligation of the vector which gives blue plaques regardless of subsequent phosphatasing. On agarose gel electrophoresis of the vector, smear band was found after GENECLEAN.

6. CONCLUSION

In this study I established protocols for DNA extraction and PCR amplification of a DNA fragment in the human FIX promoter. Unfortunately, sequencing and cloning experiments were unsuccessful. Consequently, the sequences of the amplified products could not be confirmed or compared with published data. It was unfortunate that the GENECLEAN Kit did not work. As a result, DNA of sufficient purity for direct sequencing was unable to obtain. Since the laboratory was just started for molecular cloning experiments it probably took a little bit longer than one expected time to establish these techniques and arrange facilities. Due to time constrains the cloning experiments were not confirmed. Further attempts should be made to establish protocols for cloning the PCR product so that sufficient DNA could be prepared and purified for sequencing reactions. More haemophilia B patients should be recruited for sequence comparison study and characterization although there are very few documented haemophilia B patients in Hong Kong.

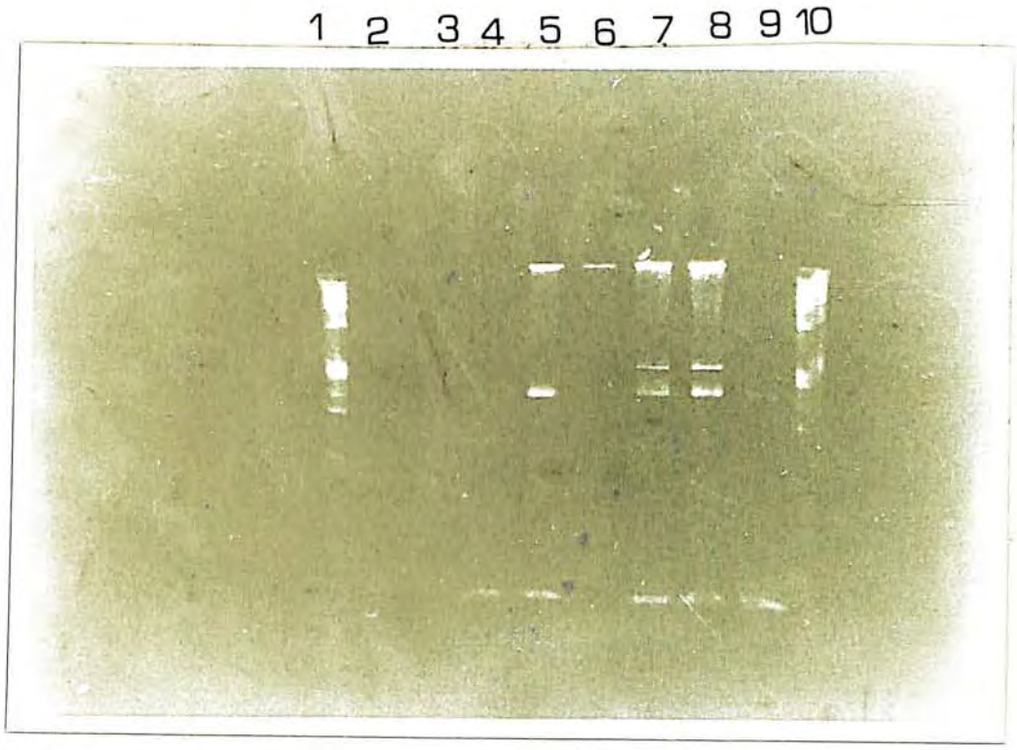

Photo-1 PCR-1

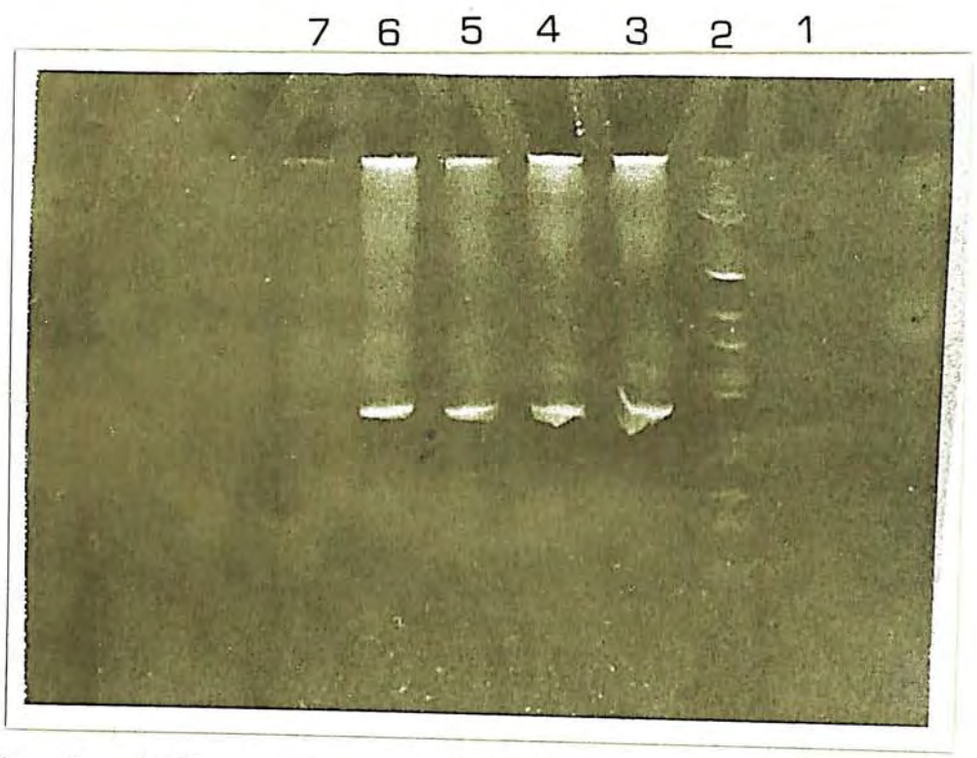

Photo-2 PCR-4

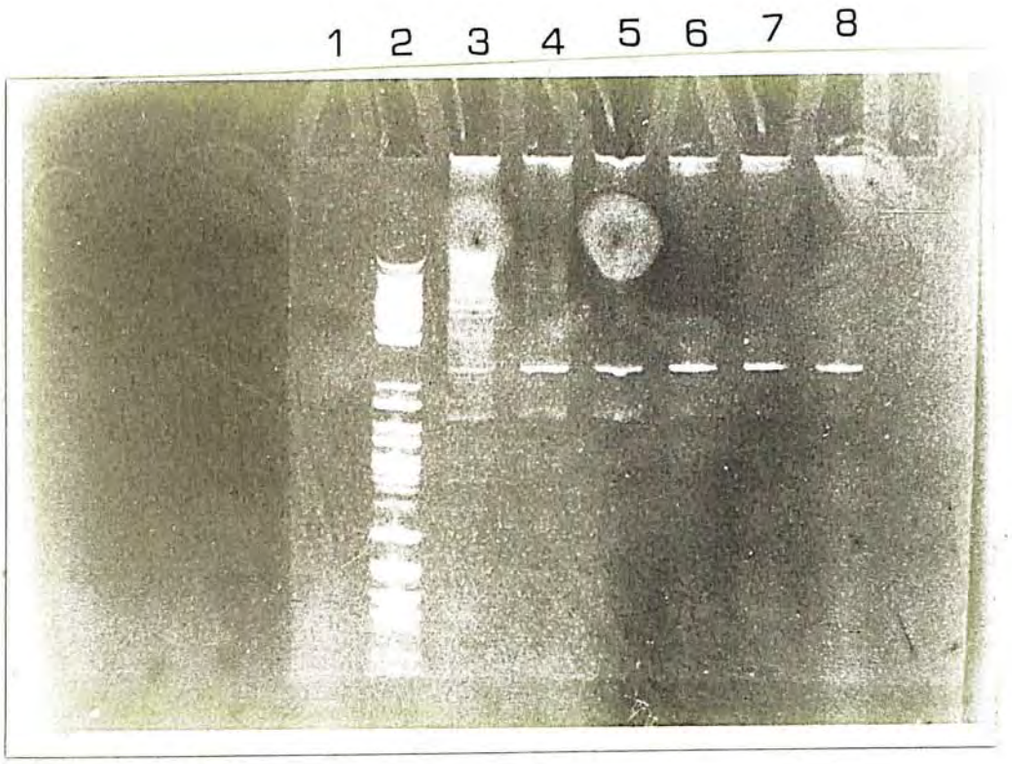

Photo-3 PCR-5

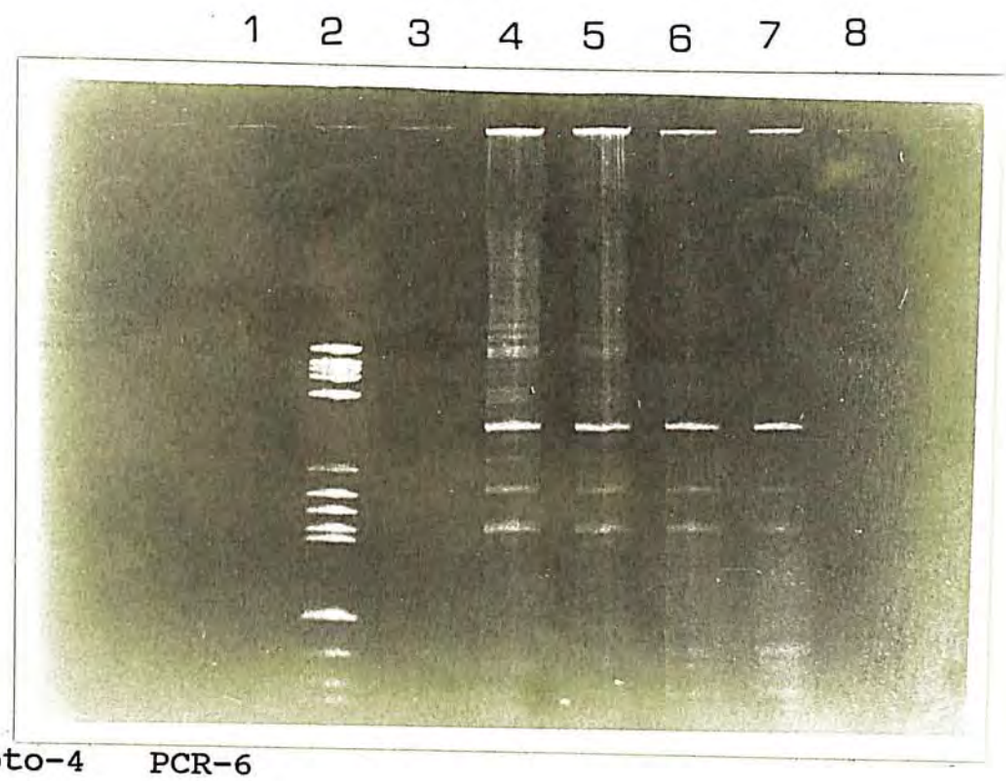

Photo-4 PCR-6

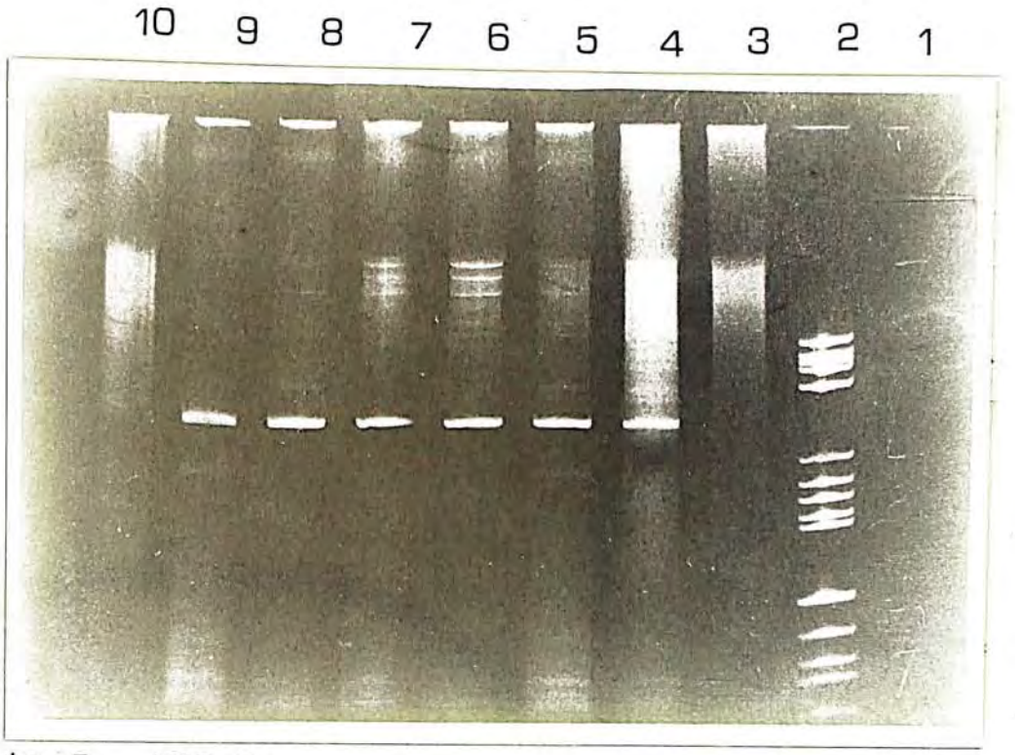

Photo-5 PCR-7

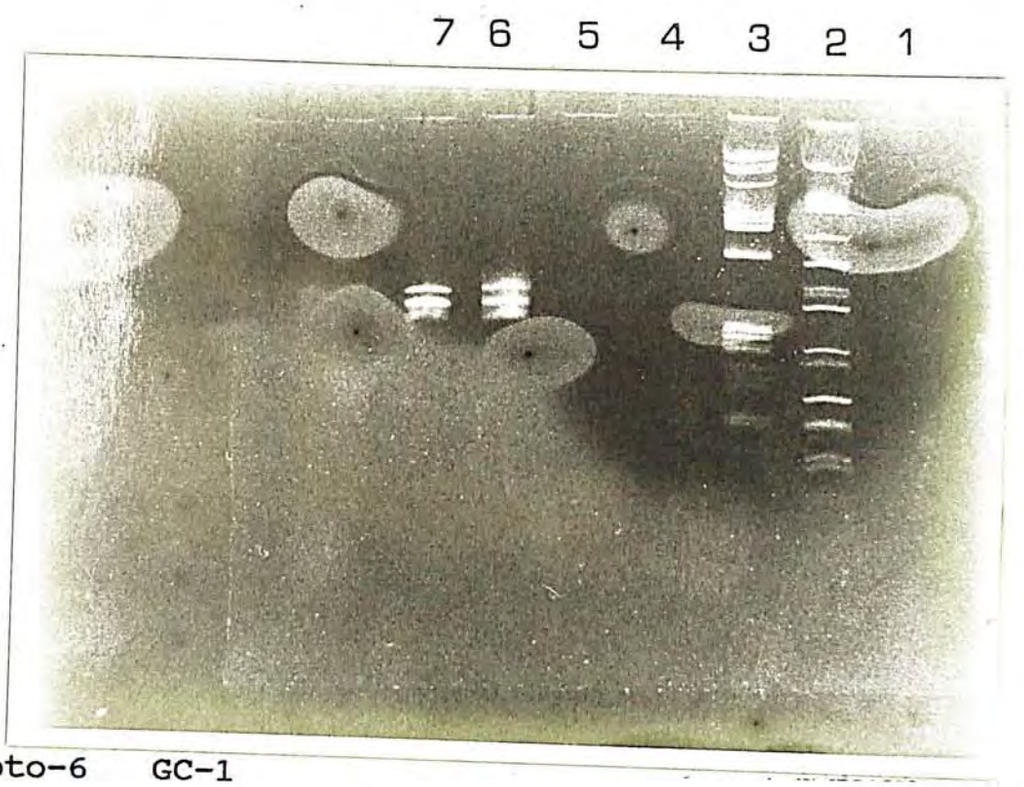

Photo-6 GC-1

Photo-7 GC-3 and PAGE-1

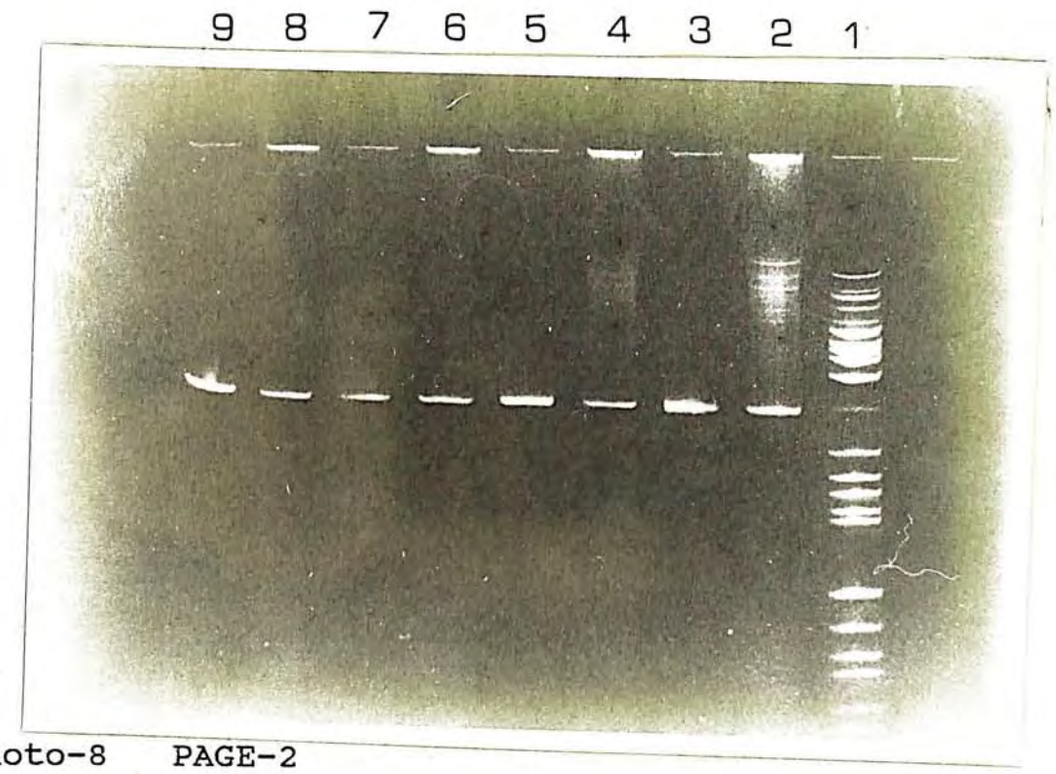

Photo-8 PAGE-2

Photo-9 Agarose gel extraction with glasswool exclusion

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